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c-Kit Expression is Rate-Limiting for Stem Cell Factor-Mediated Disease Progression in Adenoid Cystic Carcinoma of the Salivary Glands^{1,2}

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Abstract

Adenoid cystic carcinoma (ACC) is an aggressive malignant neoplasm of the salivary glands in which c-Kit is overexpressed and activated, although the mechanism for this is as yet unclear. We analyzed 27 sporadic ACC tumor specimens to examine the biologic and clinical significance of c-Kit activation. Mutational analysis revealed expression of wild-type c-Kit in all, eliminating gene mutation as a cause of activation. Because stem cell factor (SCF) is c-Kit's sole ligand, we analyzed its expression in the tumor cells and their environment. Immunohistochemistry revealed its presence in c-Kit-positive tumor cells, suggesting an activation of autocrine signaling. We observed a significant induction of ERK1/2 in the cells. SCF staining was also found in other types of non-cancerous cells adjacent to tumors within salivary glands, including stromal fibroblasts, neutrophils, peripheral nerve, skeletal muscle, vascular endothelial cells, mucous acinar cells, and intercalated ducts. Quantitative PCR showed that the top quartile of c-Kit mRNA expression distinguished ACCs from normal salivary tissues and was cross-correlated with short-term poor prognosis. Expression levels of SCF and c-Kit were highly correlated in the cases with perineural invasion. These observations suggest that c-Kit is potentially activated by receptor dimerization upon stimulation by SCF in ACC, and that the highest quartile of c-Kit mRNA expression could be a predictor of poor prognosis. Our findings may support an avenue for c-Kit-targeted therapy to improve disease control in ACC patients harboring the top quartile of c-Kit mRNA expression.

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Introduction

Adenoid cystic carcinoma (ACC) is the second most common malignant salivary gland tumor [1–3]. It arises in the major and minor salivary glands, as well as in the seromucinous glands of the upper respiratory tract, and can also occur in other bodily sites with exocrine glands, including the breast and lung. It is biphasic, composed of duct-type epithelial cells and myoepithelial cells, and forms three distinctive microscopic patterns that are categorized as predominantly tubular, cribriform, or solid. Among these three histologic subtypes, the solid form tends to have the highest recurrence rate and the worst long-term prognosis.

ACC grows slowly with extensive local spread. Perineural invasion along small and large nerves is common and often leads to pain, numbness, and paralysis. In the head and neck, ACC often spreads

into vital structures, including the brain. Although short-term survival is high, almost half of all patients develop metastases or die of complications of local recurrences within 10–20 years of diagnosis.

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Even patients who achieve local tumor control can develop distant metastases ten or more years after initial therapy. Thus, ACC is considered to be a systemic disease with an unpredictable, unrelenting course. Unfortunately, surgery, chemotherapy, and radiation therapy provide little improvement in survival. Thus, an effective therapy is urgently needed [3–5]. Possible molecular targets include the transmembrane receptor tyrosine kinases (RTKs).

c-Kit (also known as CD117) is an RTK encoded by the *KIT* gene [6]. Recent studies have demonstrated that overexpression of c-Kit occurs in almost all ACCs [3–5,7,8]. In contrast, c-Kit expression is seldom increased in other head and neck tumors. For this reason, c-Kit expression is often used as a diagnostic pathology aid for ACC. Furthermore, an analysis of protein phosphorylation of primary ACC tumors recently showed that c-Kit was phosphorylated and activated [9], although the mechanism underlying this activation remains unclear [3,5]. Chromosome copy number gains at the *KIT* loci have been found in only a small subset of ACC tumors [10], and the majority of ACCs express wild-type c-Kit [11], although we recently found inactivating c-Kit mutations in 2 of 17 ACC cases [3].

Given that c-Kit mutations in ACC are rare, c-Kit is likely to be activated by receptor dimerization upon stimulation by stem cell factor (SCF), its sole ligand [6]. SCF mRNA has been shown to be present in tumor and normal salivary tissues [9]. Once c-Kit is activated, diverse intracellular responses are induced through signaling cascades such as the phosphoinositide-3 kinase and mitogen-activated protein kinase pathways. This process contributes to numerous phenomena [6]. For example, c-Kit activation is important for a variety of normal physiologic processes, including hematopoiesis, spermatogenesis, and the growth and migration of melanocytes [3,5,6].

A recent report found that c-Kit expression was correlated with poor 3-year outcomes in ACCs, while epidermal growth factor receptor (EGFR) expression was correlated with a better 3-year outcome [12]. This finding warrants investigation of c-Kit inhibitors for potential therapeutic use. However, the data regarding the impact of c-Kit inhibition on ACC are conflicting. Two recent case reports suggested that imatinib mesylate (Gleevec) inhibits the growth of ACC [13,14]. In contrast, a Phase II clinical trial with the same drug induced no significant response in 27 patients with ACC, despite high c-Kit expression levels in their tumors [15]. These results suggest that reducing c-Kit activity may not be sufficient to inhibit ACC's progression. Nonetheless, c-Kit may play a key role in local invasion and distant metastasis by accelerating mobilization of tumor cells. In melanocytes, constitutive activation of c-Kit signaling promotes cell migration, but does not significantly contribute to melanogenesis and proliferation [16].

The objective of this study was to determine the expression of SCF in ACC tumor cells, and/or the tumor environment, and to investigate the clinical and biologic significance of c-Kit activation. We propose a potential role of SCF for c-Kit activation based on its tissue distribution and cell type-specific expression in ACC.

Materials and Methods

Tumor Samples

We obtained 27 ACC tissue samples from the University of California, San Francisco (UCSF), Anatomic Pathology archives. In addition, representative normal salivary tissue samples were chosen from five of the ACC patients: cases 2, 14, 16, 19, and 22 in Table. Institutional review board approval was obtained and UCSF guidelines for handling human tissue were followed. Slides were

reviewed to determine tissue suitability for genomic analysis and gene expression analysis and to determine the histologic tumor pattern (tubular, cribriform, or solid).

Immunohistochemistry

To examine c-Kit, SCF, or active ERK1/2 protein expression in ACC tumors, we performed immunohistochemistry (IHC) on unstained sections with antibody-based staining kits for c-Kit (104D2; Dako, Carpinteria, CA), SCF (C19H6; Cell Signaling Technology [CST], Danvers, MA), Phospho-p44/42 ERK1/2 (D13.14.4E; CST), and a rabbit isotype control (3900; CST). The staining procedure has been described [3]. c-Kit, SCF, and P-ERK1/2 staining was visually estimated by a head and neck pathologist (AvZ). Assessment included the percentage of tumor cells staining positive and the intensity of staining on a five-point scale from negative (0) to very strongly positive (4+).

Mutation Analysis

Genomic DNA from each case of ACC was isolated from formalin-fixed paraffin-embedded (FFPE) tissue sections with a QIAampDNA FFPE Tissue kit (Qiagen, Valencia, CA) [3]. DNA samples were amplified by PCR with the primer sets listed below and proofreading capability platinum Taq DNA polymerase (Life Technologies, Carlsbad, CA). Direct sequencing of PCR products was performed at the UCSF Genomics Core Facility with ABI BigDye v3.1 dye terminator sequencing chemistry (Applied Biosystems, Carlsbad, CA), an ABI PRISM 3730xl capillary DNA analyzer (Life Technologies, Carlsbad, CA), and Mutation Surveyor v2.5 (SoftGenetics, State College, PA). We used the following oligonucleotide primer sequences for detecting the *KIT* gene:

5'-CAGATTCTGCCCTTTGAACTTG-3' and 5'-AAAAAGC CACATGGCTAGAAAAA-3' (Exon 8; 392 bp);
5'-TAAAAGTATGCCACATCCCAAG-3' and 5'-CATGGT CAATGTTGGAATGAAC-3' (Exon 9; 368 bp);
5'-TCCAGAGTGCTCTAATGAC-3' and 5'-AGGTGGAA CAAAACAAAGG-3' (Exon 11; 292 bp);
5'-AAGATGCTCAAGCGTAAGTTC-3' and 5'-AAGCAGTT TATAATCTAGCATTGCC-3' (Exon 13; 302 bp); and
5'-GTGAACATCATTCAAGGCGT-3' and 5'-CCTTTGCAG GACTGTCAAGCA-3' (Exon 17; 336 bp).

TaqMan Quantitative PCR Assay

Gene expression was analyzed in triplicate with TaqMan quantitative PCR. Total RNA was isolated with RNeasy kits (Qiagen, Valencia, CA) from FFPE tumor tissue sections composed of at least 70% tumor cells. cDNA from 500 ng of total RNA was synthesized with an RT First Strand Kit (Life Technologies, Carlsbad, CA). cDNA (5 ng) was mixed with RT qPCR master mixes, and aliquots were placed with gene-specific primer sets. We used the following TaqMan assays (all from Life Technologies): KIT (Hs00174029_m1), SCF (Hs00241497_m1), and EGFR (Hs01076078_m1). Expression levels normalized to endogenous GAPDH were determined by real-time PCR and analyzed at the UCSF Core noted above. Statistical analyses were performed and graphs made with Microsoft Office Excel and XL-Stat (Addinsoft, New York, NY). Statistical comparisons between data sets were made with two-tailed Student's *t* tests and Wilcoxon tests according to the manufacturers' instructions, with *P* < .05 considered significant. (Wilcoxon testing, a hypothesis assessment that compares survival distributions in two samples, is more sensitive than log-rank testing

Table. Clinicopathologic Features of ACC Samples

Case	Gender	Age	Tumor sites	Histology	c-Kit		SCF		P-ERK1/2	
					Positive luminal cells	Staining intensity	Positive tumor cells	Staining intensity	Positive tumor cells	Staining intensity
1 [†]	M	78	Base of tongue	Solid + tubular	100%	3+	100%	1+	20%	2+
2	F	64	Mandibular mucosa	Tubular + cribriform	100%	1-3+	100%	1-2+	2%	3+
3	M	46	Maxillary sinus	Tubular + cribriform	100%	2-3+	100%	1+	50%	2-3+
4	M	42	Maxillary sinus	Tubular	100%	2-3+	60%	1-2+	20%	2+
5	M	59	Maxillary sinus	Tubular + cribriform	100%	1-3+	100%	1-2+	10%	2-3+
6	F	68	Maxillary sinus	Tubular + cribriform	100%	3+	80%	1+	50%	2-3+
7	F	75	Maxillary sinus	Solid + tubular	100%	2-3+	50%	1-2+	2%	2-3+
8	F	36	Maxillary sinus	Solid + tubular	100%	3+	100%	1+	1%	3+
9	M	41	Maxillary sinus	Tubular	100%	3+	100%	1+	40%	2-3+
10	F	42	Maxillary sinus	Tubular	100%	3+	100%	1-2+	70%	2+
11 [†]	F	64	Maxillary sinus	Tubular + cribriform	100%	2-3+	100%	1-2+	85%	2-3+
12	F	66	Nasal cavity	Solid + cribriform	100%	2-3+	80%	1+	20%	2-3+
13	F	44	Nasopharynx	Cribriform	100%	2-3+	100%	1+	50%	2+
14	F	91	Parotid gland	Solid	100%	3+	100%	1+	0%	0
15 [†]	F	44	Parotid gland	Solid + tubular	100%	2-3+	100%	1+	10%	3+
16 [†]	M	33	Parotid gland	Tubular + solid	100%	3+	100%	1-2+	15%	2-3+
17	F	54	Parotid gland	Tubular + solid	100%	3+	10%	1+	20%	2-3+
18	F	60	Parotid gland	Solid + tubular	100%	3+	80%	1+	20%	2-3+
19	M	66	Sublingual gland	Cribriform	100%	3+	100%	1+	30%	2-3+
20	F	73	Sublingual gland	Cribriform + tubular	100%	2-3+	40%	1+	15%	2-3+
21 [†]	M	78	Submandibular gland	Tubular + cribriform	100%	3+	100%	1-2+	50%	2+
22	M	59	Submandibular gland	Cribriform + tubular	100%	2-3+	100%	1+	20%	2-3+
23 [†]	M	54	Submandibular gland	Tubular	100%	3+	100%	1+	30%	2-3+
24	F	44	Submandibular gland	Cribriform + tubular	100%	2-3+	90%	1-3+	2%	3+
25 [†]	F	51	Submandibular gland	Cribriform + tubular	100%	2-3+	90%	1-2+	55%	3+
26 [†]	F	70	Submandibular gland	Tubular + solid	100%	2-3+	100%	1+	35%	3+
27	M	68	Tongue	Cribriform	95%	1-3+	20%	2+	0%	0

Control normal salivary tissues were chosen from cases 2, 14, 16, 19 and 22.

[†] Perineural invasion was found in cases 1, 11, 15, 16, 21, 23, 25 and 26.

when the ratio of hazards is higher at earlier rather than later survival times). Kaplan-Meier plots were generated and patients were divided into groups on the basis of gene expression.

Statistical Power Analysis

Statistical power analysis for determining the sample size effective for the result was performed by using Time to an Event, a sample-size calculator (http://hedwig.mgh.harvard.edu/sample_size/time_to_event/para_time.html). All statistical tests were two-sided and conducted at the .05 significance level.

Median survival was defined as the time after which 50% of patients with ACC were living. The median survival ratio (>1) was calculated by dividing one group's smallest median survival time by the other group's smallest median survival time.

Results

Tumor Characteristics

Table summarizes the clinical attributes of the patients in whom the 27 ACC tumor samples were obtained. All tumors had arisen sporadically; 16 occurred in women; and the median age at presentation was 58 years (range, 33 to 91 years). Tumors arose at the following sites: maxillary sinus (9 tumors), submandibular gland (6 tumors) or (6), parotid gland (5 tumors) or (5), sublingual gland (2 tumors) or (2), and one each in the nasal cavity, mandibular mucosa, nasopharynx, base of tongue, and tongue. Tumors were classified by morphologic subtype: tubular (4 tumors) or (4), cribriform (3 tumors) or (3), solid (1 tumor) or (1), combined cribriform and tubular (10 tumors) or (10), combined solid and tubular (8 tumors) or (8), and combined cribriform and solid (1 tumor) or (1).

Expression of c-Kit Protein is Elevated in Sporadic ACC Tumors

We performed hematoxylin and eosin (H&E) staining (Figure 1A) and antibody-based IHC for c-Kit on tumor sample sections (Figure 1B [case 17] and Supplemental Figures 1B [case 2] and 1F [case 7]). Mast cell staining was a positive internal control with the antibody (data not shown). c-Kit staining was estimated as described in Methods, and Table shows our results. c-Kit expression occurred in the inner luminal (duct-type epithelial) cells of all the tumors (see Figure 1B and Supplemental Figure 1B and F), as reported previously [3].

Sequencing of KIT Gene Exons in the 27 ACC Tumors

In searching for genomic alterations, we examined exons 8, 9, 11, 13, and 17, which encode domains for dimerization (exons 8 and 9), the juxtamembrane region (exon 11), and protein kinase activity (exons 13 and 17). We chose them for this study because gain-of-function mutations are recurrently found in these regions in other neoplasms [6]. We performed direct sequencing of each exon's PCR product. Each sample was confirmed by at least three different sets of mutation analyses. No missense, frameshift, nonsense, synonymous missense, or splice mutations were detected.

SCF Expression in ACC Cells and Other non-Cancerous Cells Adjacent to Tumors Within the Salivary Glands

In light of the results of our mutational analysis, we hypothesized that c-Kit was activated by receptor dimerization upon stimulation by SCF and used IHC to determine levels of SCF protein in the salivary glands in tumor sample sections (Figure 1, C and D [case 17] and Supplemental Figure 1C [case 2] and 1G [case 7]). SCF is present not only as a secreted protein but also in membrane-bound form [6],

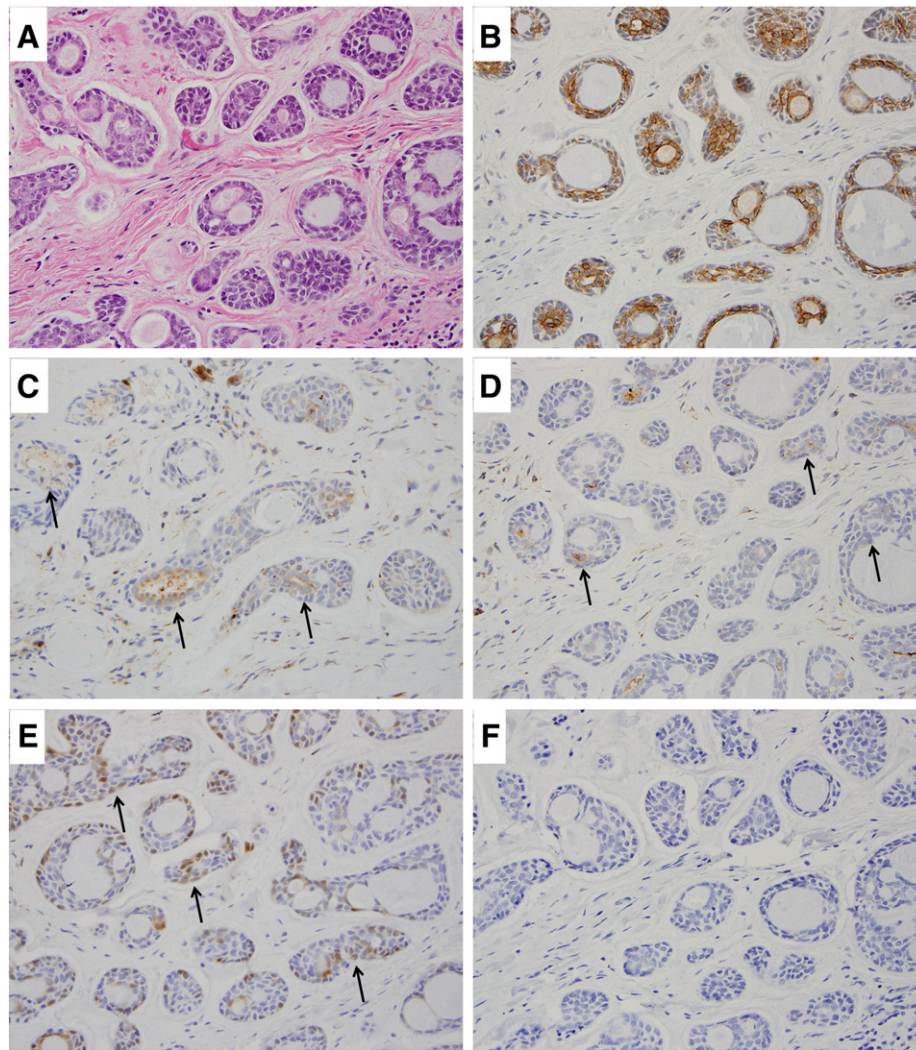


Figure 1. Expression of c-Kit, SCF, and P-ERK1/2 in ACC of the salivary glands. (A) H&E staining. (B)–(F). Immunohistochemistry with antibodies to c-Kit (B), SCF (C and D), phospho-p44/42 ERK1/2 (E); antibody isotype control (F). SCF staining was largely in the duct-type epithelial component in the tumors (C and D; indicated by black arrows) where c-Kit was predominantly elevated (B). We observed a significant induction of ERK1/2 activity in the cells (E; black arrows). The staining intensity scales are as follows; c-Kit (B: 2-3 +), SCF (C and D: 1 +), and P-ERK1/2 (E: 2 +).

although the limitations of IHC resolution could not provide a clear distinction. Table summarizes our results. The vast majority of the tumors expressed SCF (Figure 1B and Supplemental Figure 1B and F); it was largely found in the duct-type epithelial component (Figure 1C) where c-Kit was predominantly elevated (Figure 1B).

We used antibody-based IHC to detect active forms of ERK1/2 on tumor sections (Figure 1E). The Ras-Raf-MEK1/2-ERK1/2 cascade is a major downstream effector-signaling pathway of RTKs, including c-Kit. Thus, SCF-induced activation of c-Kit would accompany active ERK1/2 expression in the inner duct-type epithelial component of the tumor cells where c-Kit was elevated. Table summarizes our results. In 17 of 27 ACCs, active ERK1/2 protein was substantially increased in more than 20% of tumor cells.

Interestingly, other types of non-cancerous cells adjacent to tumors within salivary glands were positive for SCF. They included stromal fibroblasts (Figure 2A and Supplemental Figure 2A and B), neutrophils (Figure 2B and Supplemental Figure 2C and D), peripheral nerve (Figure 2, C–E and Supplemental Figure 2E),

skeletal muscle (Figure 2F and Supplemental Figure 2F), vascular endothelial cells (Figure 2G), and mucous acinar cells and intercalated ducts (Figure 2H). Strong immunoreactivity to the SCF antibody was found in neutrophils and peripheral nerve (Figure 2, B and D). In addition, Figure 2E shows that staining for SCF highlights a peripheral nerve with a tumor wrapping around the nerve bundle, creating a targetoid pattern.

The Top Quartile of c-Kit mRNA Expression Distinguishes ACCs from Normal Salivary Tissues

We investigated whether mRNA expression of c-Kit and SCF was also elevated in ACC (Figure 3, A and B), and also included EGFR because it has been implicated in the development of ACC ([17,18]; Figure 3C). mRNA was isolated from FFPE sections as described above, and quantitative PCR performed. Figure 3A shows that c-Kit mRNA expression was elevated in ACC, with the relative expression increased by 1.88 ($P < .05$) over the average of

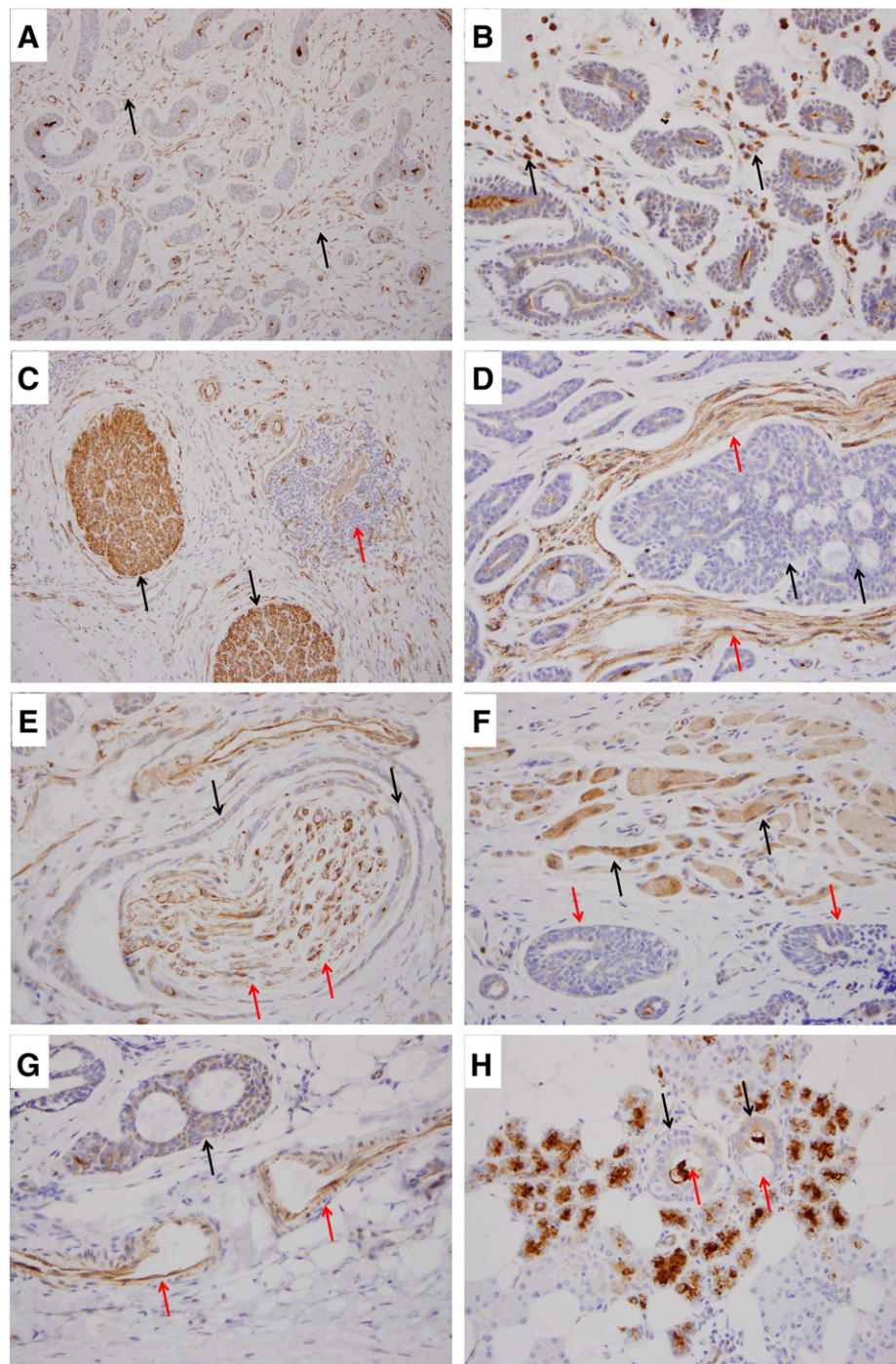


Figure 2. Immunohistochemical staining with antibodies to SCF in ACC cells and non-cancerous cells in the salivary glands (A-H). (A) Stromal fibroblasts (black arrows). (B) Neutrophils around tumor (black arrows). (C) Nerve twigs (black arrows) and lymphocytes (red arrows). (D) Tumor (black arrows) invading nerve (red arrows). (E) Targetoid pattern of tumor cells (black arrows) in perineural (red arrows) invasion. (F) Skeletal muscle (black arrows) and tumor (red arrows). (G) Tumor (black arrows) and endothelial cells (red arrows) in blood vessels. (H) Mucous acinar cells (black arrows) and intercalated ducts (red arrows).

normal samples. The top quartile of mRNA expression of c-Kit particularly distinguished ACCs from normal salivary tissues. In contrast, the expression levels of SCF and EGFR mRNA showed a broad range, which overlapped with those in normal tissue (Figure 3, B and C) and showed no significant difference ($P > .05$) from ACC samples.

Expression Levels of SCF and c-Kit are Highly Correlated in Cases With Perineural Invasion

Given that SCF-mediated c-Kit activity is important for local invasion and metastasis, we determined the strength of correlation between SCF and c-Kit mRNA expression in the presence (cases 1, 11, 15, 16, 21, 23, 25 and 26; Table) or absence of perineural invasion (PNI). We

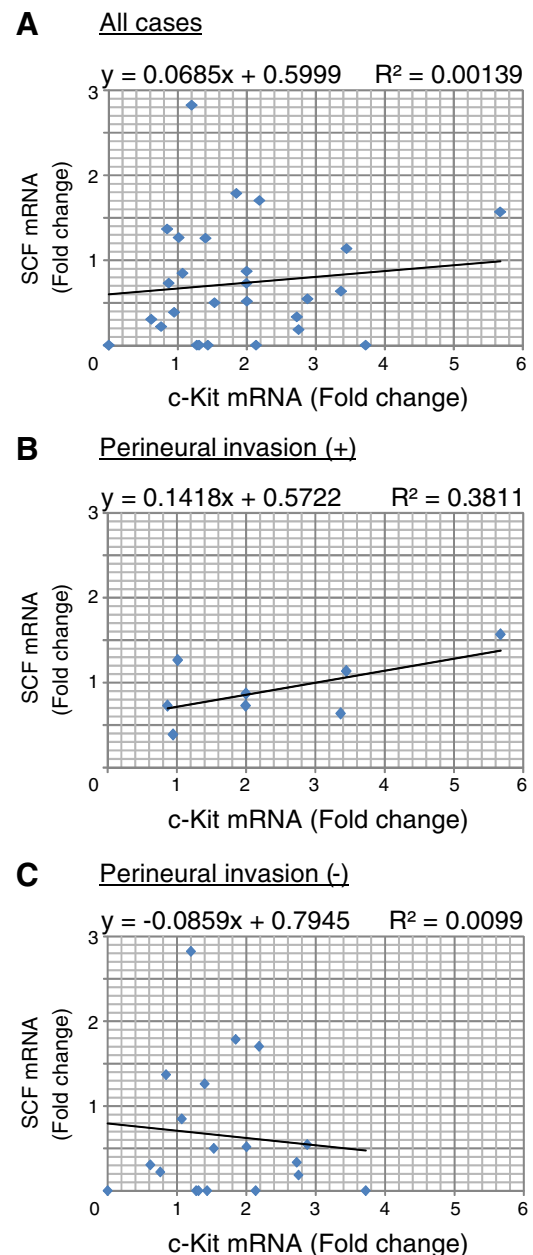
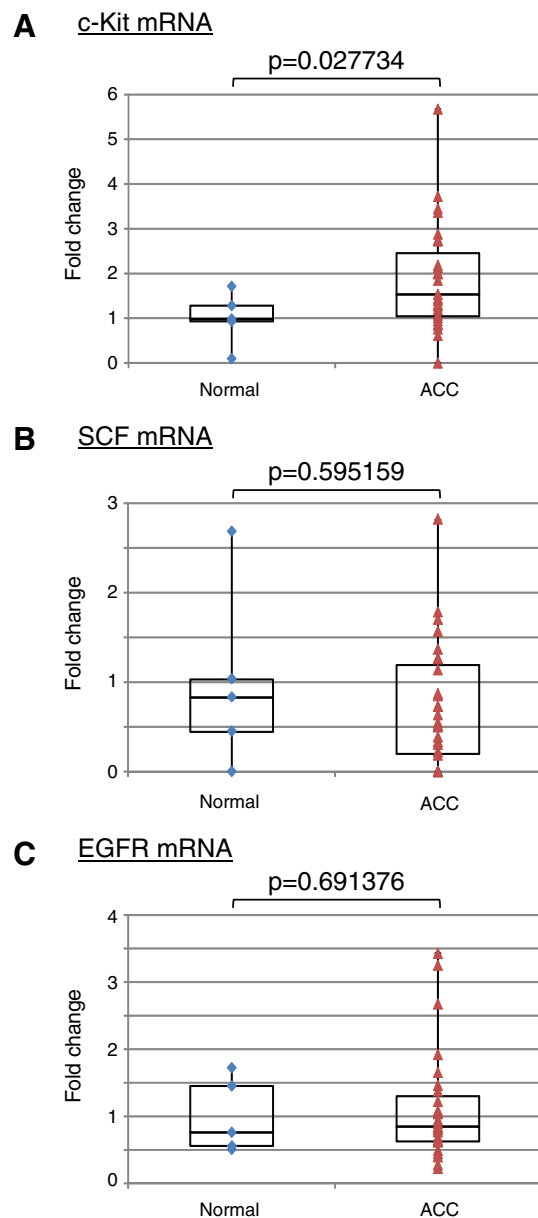


Figure 3. Box plot analyses of relative gene expression of c-Kit (A), SCF (B), and EGFR (C) in ACC tumor samples and normal tissue. Each box represents the quartile distribution range (25–75%) with the median shown by a black horizontal line. The range and individual cases are displayed as a black vertical line and scatter dots, respectively. The y-axis indicates the fold-change relative to the average of normal samples' gene expression. Statistical comparisons between data sets were performed with two-tailed Student's *t* tests, with $P < .05$ considered significant.

Figure 4. Expression levels of SCF and c-Kit in cases with perineural invasion. (A)–(C) Scatter plot analysis for the strength of a correlation between SCF and c-Kit mRNA expression in the presence or absence of perineural invasion. (A) All cases. (B) Cases with perineural invasion. (C) Cases without perineural invasion. Trend lines are included. Trend line equations and R-squared values were calculated separately.

generated scatter plots with trend lines to show correlations (Figure 4, A–C). Trend line equations and R-squared values were calculated with Microsoft Excel and are displayed atop each chart.

Expression levels of SCF and c-Kit were highly correlated in the 8 cases with PNI (Figure 4B); R-squared values (0 to 1 range) were 0.381 ($R = 61.73\%$). In contrast, SCF and c-Kit expression correlated poorly in the absence of PNI (Figure 4C; R-squared values 0.0099 [$R = 9.94\%$]).

The Highest Quartile of c-Kit mRNA Expression Predicts Poor Prognosis in Salivary ACC Patients

To determine the biologic and prognostic significance of c-Kit, SCF, and EGFR mRNA expression, we performed overall survival analysis by generating Kaplan-Meier plots with Wilcoxon testing (Figure 5, A–D). We divided our ACC cohort into 2 groups according to gene expression scores (group 1: above the median; group 2: below it; Figure 5, A, C, and E) and also created groups whose expression values were in the

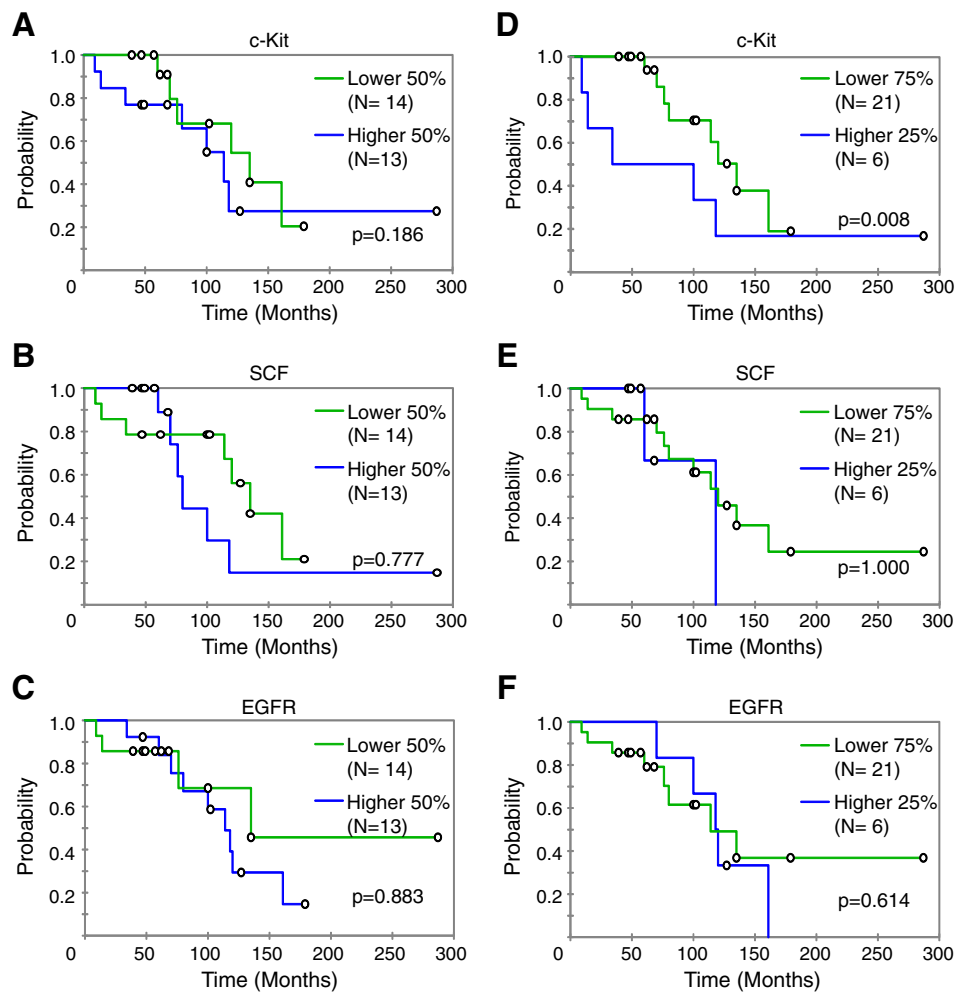


Figure 5. Kaplan-Meier curves showing overall survival rates of ACC patients according to mRNA expression of c-Kit (A and D), SCF (B and E), or EGFR (C and F). The ACC cohort was divided into groups according to relative gene expression. Panels A to C compare patients with expression in the top half overall (blue line) and the lower half (green line) for each gene. Panels D to F compare expression in the higher quartile (blue line) and lower three quartiles (green line). Circles identify censored data. Statistical comparisons between data sets were made with Wilcoxon tests and each P value is displayed, with $P < .05$ considered significant.

highest or lowest quartiles (Figure 5, B, D and F). c-Kit expression correlated with survival (Figure 5, A and D). Specifically, the subset with the highest c-Kit gene expression (top quartile), which did not overlap with the gene expression in normal tissue (Figure 3A), had the poorest survival ($P = .008$).

To determine whether our sample size in the analysis provided significance to this result, we performed a statistical power analysis as described in Methods [19]. The total number of 27 cases corresponded to a power of 0.87, providing confidence to this result, where a power of ≥ 0.80 (equivalent to ≥ 22 total cases) is sufficient to detect a large difference between two groups. In contrast, we did not find a significant correlation between survival and expression of SCF and EGFR (Figure 5, B, C, E, and F).

Discussion

c-Kit is overexpressed and phosphorylated in sporadic ACCs without gene mutations [5]. The presence of SCF mRNA in tumor and normal salivary tissue has been reported as a potential mechanism for c-Kit activation in ACC [9]. However, it is not clear how SCF is expressed or to what extent it contributes to c-Kit activation. The

goal of this study was to characterize the pattern of SCF protein expression in ACC tumor cells, and/or in the tumor environment, and to examine the clinical and biologic significance of c-Kit activation in ACC patients.

c-Kit is an oncogene [6]. Gain-of-function mutations in it occur in a range of human cancers and are advantageous for tumor growth, survival, and disease progression. For example, c-Kit mutations are often found in mast cell leukemia and gastrointestinal stromal tumors (GIST; 5, 6). However, gene mutations were not a cause of c-Kit activation in our cohort of 27 ACCs studied here. Our results confirmed studies from other laboratories [7,11].

We investigated whether ACC cells expressed c-Kit's ligand, SCF. SCF was present not only in the tumor cells, which could mediate autocrine signaling, but also in other types of cells adjacent to the salivary glands. These cells might facilitate paracrine signaling [20]. In particular, SCF expression was highest in nerve cells in the tumor microenvironment. Peripheral nerves appear to release SCF into the neural space, where it could act as a chemo-attractant and growth factor critical for ACC. This observation may explain why ACC has a strong tendency to infiltrate into neural structures and spread perineurally, and

why the tumor cells are frequently distributed around the nerve bundles in a targetoid pattern. Moreover, we speculate that SCF may induce c-Kit expression through a positive-feedback loop, a possibility supported by our observation that expression levels of SCF and c-Kit were highly correlated in the cases with perineural invasion. This finding is in agreement with a recent report: c-Kit-negative PC3 prostate cancer cells gained c-Kit expression when the cells developed metastasized bone tumors in xenograft mice, where the bone marrow stromal cells expressed SCF [21]. The study may offer a valuable clue about why slow-growing ACCs become aggressive when the tumors invade the neural space or metastasize to bone.

In this work, we performed phospho-ERK1/2 IHC simply as a way to facilitate analysis. Our choice of this approach was not intended to imply that ERK1/2 is phosphorylated only by SCF-mediated c-Kit activation. Moreover, the results were variable between cases likely owing to the nature of antigenicity of phosphorylated protein. A recent study showed that phosphorylated-ERK1/2 in primary tumors was largely degraded in the process of formalin-fixation [22]. The extreme rarity of ACC limits the fresh tissue donor pool. In addition, phospho-c-Kit IHC with FFPE samples is not yet established. Thus, in light of these limitations, we believe that using phospho-ERK1/2 IHC with FFPE samples is the most practical approach for accomplishing our purpose. There was a substantial increase of active ERK1/2 protein in more than 20% of ACC tumor cells. We found that immunoreactivity was greater in the outer myoepithelial cells than in the inner duct-type epithelial cells. The difference could be attributed to the characteristic difference between two cell types in ACC. c-Kit protein is specifically elevated in duct-type epithelial cells, whereas EGFR expression is limited to the myoepithelial cells [12]. Moreover, a differentiation marker p63 is predominantly found in the myoepithelial but not duct-type epithelial component [23]. Thus, ERK1/2 activation appeared to be accelerated in differentiated cells in ACC.

In this paper, we found that the highest quartile of c-Kit mRNA expression was cross-correlated with short-term poor prognosis. Because quantitative PCR is sensitive, reproducible and reliable for determining the level of c-Kit mRNA, this gene expression analysis may have a larger potential to identify the patients more likely to benefit from c-Kit-targeted therapies in ACC [24,25]. These therapies may include targeting c-Kit protein or upstream molecules that regulate it.

It has been suggested that c-Kit is a downstream transcriptional target of MYB, which is activated by gene fusion with nuclear factor nuclear factor I/B (NFIB) in roughly half of ACC tumors [26,27]. However, discrepancies in its cell-type-specific expression have led to questions about the role of MYB for c-Kit transactivation in ACC. In recent studies, MYB protein was elevated in myoepithelial cells, whereas c-Kit expression was limited to the duct-type epithelial cells [12,28,29]. Further investigation is necessary, but c-Kit appears to be regulated by a mechanism other than MYB activation in ACC tumors. As a consequence, c-Kit may not be a useful biomarker to measure response to MYB inhibitors in salivary tumors.

Imatinib is used to treat GISTs, which harbor oncogenic c-Kit [6]. The initial response to the drug is usually dramatic. Unfortunately, most GISTs develop secondary *KIT* mutations during treatment, resulting in drug resistance and subsequent recurrence. Nonetheless, when imatinib is used as an adjuvant after surgical resection of localized primary GISTs, the treatment offers long-term survival and may result in a cure [30]. A similar adjuvant-based approach may improve outcomes for a subset of ACC patients bearing the top

quartile of c-Kit mRNA expression, and antibody-based c-Kit targeted therapies could be also applicable [31,32].

In summary, c-Kit was shown to be potentially activated by receptor dimerization upon stimulation by SCF in ACC. We determined the pattern of SCF expression in the tumor cells and other types of non-cancerous cells in salivary glands. We also showed that the highest quartile of c-Kit mRNA expression distinguished ACCs from normal salivary tissues and was a potential biomarker to predict short-term poor prognosis in ACC patients. Given that there are no validated ACC cell lines that have not been immortalized, development of authenticated ACC cell lines is an important next step to substantiate further the clinical usefulness of our findings here [2].

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.tranon.2014.07.006>.

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